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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Development of human ovarian cancer depends, in part, on formation of an adequate blood supply. Tumor angiogenesis is essential for cancer growth, and vascular endothelial growth factor (VEGF) is critical in stimulating growth of vascular endothelial cells. VEGF is produced by ovarian cancers, and VEGF secretion is markedly increased in ovarian cancers with HER-2 oncogene overexpression. Herceptin, an antibody to HER-2 receptor, has direct antitumor effects, but the antireceptor antibody also elicits a significant reduction in VEGF secretion from ovarian cancer cells,and, thereby, retards ovarian tumor-associated angiogenesis. More complete suppression of angiogenesis may be elicited by treatments that synergistically suppress blood vessel proliferation, such as squalamine, an angiostatic steroid approved by the FDA as an orphan drug candidate for treatment of ovarian cancer. In studies with human ovarian cancer cells *in vivo*, squalamine elicits antitumor activity by suppressing the angiogenuc action of several vascular growth factors including VEGF, an effect that may be due to squalamine-induced blockade of MAP kinase activation. Ongoing work evaluates the efficacy of squalamine alone and combined with other antitumor therapies, including cisplatin, carboplatin and Herceptin, in suppressing growth of human ovarian cancers with and without HER-2 oncogene overexpression.

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INTRODUCTION

Ovarian cancer is the most deadly gynecologic malignancy. About 26,500 women are diagnosed with this cancer each year and have an overall 5-year survival rate of only 47% (1, 2). For most patients, surgery alone does not cure the cancer due to the spread of tumors beyond the confines of the ovary, and management in the clinic often requires use of toxic chemotherapy regimens. The progressive growth of ovarian cancer depends, in part, on the formation of an adequate blood supply, and tumor angiogenesis has been reported to have prognostic significance in epithelial ovarian cancer (3). Therapy directed toward the vasculature of solid tumors is now being pursued as an important new direction in cancer treatment, because avascular tumors exhibit limited growth (4,5) and tumor aggressiveness and metastatic potential commonly correlate with tumor vascularity (6).

Vascular endothelial growth factor (VEGF) is produced by most solid tumors and elicits a mitogenic effect on tumor-associated endothelial cells (7, 8). VEGF binding to receptor tyrosine kinases triggers activation of downstream signaling enzymes, including p42/p44 MAP kinase, which, in turn, regulate gene expression and specific endothelial cell responses including proliferation, migration, differentiation and apoptosis (9,10). VEGF plays an important role in progression of ovarian cancer (3, 11, 12), and the ability of VEGF to increase vascular permeability (7, 8, 13) may also promote formation of malignant ascites (14). Growth factor pathways, such as those dependent on the HER-2 receptor, appear to up-regulate VEGF production in some solid tumors (15). Since HER-2 receptor is overexpressed in a significant number of ovarian cancers (16, 17), it may also play a role in promoting further growth of ovarian malignancy by increasing VEGF-dependent tumor angiogenesis.

Squalamine, a sterol from tissues of dogfish shark (18), has significant anti-angiogenic and antitumor activity in laboratory models of brain, breast and lung cancer (19-22). Squalamine is a 7,24-dihydroxylated 24-sulfated cholestane steroid conjugated to spermidine at C-3, and it blocks endothelial cell growth and exhibits inhibitory activity in chick embryo chorioallantoic membrane and rabbit corneal micropocket assays (19, 20,23). This anti-angiogenic agent may have good potential for clinical application because it inhibits endothelial cell proliferation induced by a wide range of growth factors, including VEGF (19). This inhibition may result, in part, from its interaction with endothelial cell surface proton pumps, thereby altering intracellular pH and impeding signaling by growth factors (24, 25). When administered as a single agent in nude mice with lung cancer xenografts, squalamine has limited antitumor activity, but the anti-angiogenic steroid significantly enhances the antitumor efficacy of cisplatin and carboplatin/paclitaxel chemotherapies (20-22). Since platinum-based treatments are often used for human ovarian cancers (1, 2), squalamine in combination with cisplatin or carboplatin was studied to assess its utility as part of a coordinated attack against human ovarian cancers and their blood supply. One additional feature we were particularly interested in was the consequence of HER-2 oncogene overexpression for squalamine modulation of growth in ovarian tumor xenografts. Amplification and/or overexpression of HER-2 oncogene in human cancers, including ovarian cancers, is often associated with poor clinical outcome (16,17), and human ovarian tumor cells with overexpression of HER-2 membrane receptor also exhibit resistance to cisplatin and carboplatin (26). We therefore examined whether the level of HER-2 expression in paired HER-2-transfected and non-transfected ovarian cancers influenced the degree of tumor growth inhibition seen with squalamine with or without concomitant platinum-based treatment.

BODY: RESEARCH PROGRESS

AIM 1) Evaluation of angiogenic activity due to HER-2 gene overexpression in human ovarian cells.

1.a. *Squalamine Does not Affect VEGF Secretion in vitro for Ovarian Cancer Cells with or without HER-2 Gene Overexpression.*

HER-2 overexpression is generally thought to lead to tumor development through its effects on promoting uncontrolled cancer cell growth. However, recent findings suggest that HER-2 may also regulate cell survival functions such as angiogenesis by promoting tumor production of VEGF (15). To explore how HER-2 may contribute to angiogenesis in ovarian cancer, we evaluated HER-2 effects on *in vitro* VEGF secretion by human

ovarian tumor cells. Parent and HER-2-overexpressing ovarian 2008 cells were incubated for 72 h *in vitro*, and secretion of VEGF into conditioned media was measured by use of established enzyme-linked immunosorbant assay (see Fig. 6 in ref. 27). Parent ovarian cancer cells show significant secretion of VEGF, and, after transfection of ovarian cells with HER-2 gene to high levels, a further increase in VEGF secretion was found. In parallel *in vitro* studies, treatment of ovarian cancer cells with squalamine elicited no significant effect on secretion of VEGF (27). Thus, HER-2 overexpression may contribute to angiogenesis through up-regulation of VEGF secretion in ovarian cancer, but squalamine is not anti-angiogenic at this step in tumor-associated angiogenesis since it does not appear to directly affect secretion of VEGF by ovarian epithelial tumor cells.

AIM 2) Assessment of biologic activities of squalamine, a newly-synthesized anti-angiogenic steroid, using human vascular endothelial cells *in vitro*.

2.a. *Squalamine Blocks VEGF-Stimulated Proliferation of Endothelial Cells in vitro.*

To assess potential biologic mechanisms for antiangiogenic and antitumor effects of squalamine noted previously, human umbilical vein endothelial cells (HUVEC) were grown *in vitro*. VEGF elicits significant proliferation of HUVEC cells by 72 h. In the absence of VEGF, squalamine has no effect on proliferation or survival of HUVEC cells. However, in the presence of VEGF, squalamine elicited a significant reduction in VEGF-induced endothelial cell proliferation (see Fig. 5 in ref. 27). This growth-suppressive effect of squalamine appears restricted to endothelial cells since the compound had no direct inhibitory effect on the proliferation of ovarian 2008 cancer cells, either with or without HER-2 gene overexpression (27).

2.b. *Squalamine Blocks VEGF-Induced Activation of MAP Kinase in vitro.*

VEGF exerts its biologic effects by binding with receptor tyrosine kinases, notably Flt-1 and Flk-1/KDR, present at the surface of endothelial cells (9). Post-receptor signal transduction regulates effects of VEGF, and proliferative effects of VEGF in endothelial cells have been associated with VEGF-induced tyrosine phosphorylation and stimulation of mitogen-activated protein kinases (MAP kinase), extracellular signal-regulated kinase ERK-1 ($p44^{MAPK}$) and ERK-2 ($p42^{MAPK}$) (9, 10). On the assumption that blockade of endothelial cell proliferation by squalamine may occur, in part, by suppression of MAP kinase signaling cascades induced by growth factors, VEGF-induced tyrosine phosphorylation of MAP kinases was assessed. As expected, VEGF promotes tyrosine phosphorylation of p42/p44 MAP kinase isoforms, with maximal effects evident by 10 min (see Fig. 7 in ref. 27). However, after administration of squalamine, the VEGF-stimulated phosphorylation of MAP kinase isoforms is significantly suppressed, especially after 30 min exposure to VEGF (27). Following success in these studies, additional work will now focus on squalamine-mediated changes in the activity of p38 MAP kinase and the association of p38 MAP kinase with F-actin formation and focal adhesion assembly, important functions in the migration and proliferation of vascular endothelial cells (9,10,25).

AIM 3) Investigation of the efficacy of squalamine alone and combined with other antitumor agents in blocking the *in vivo* growth and progression of human ovarian cancer xenografts in nude mice.

3.a. *Squalamine and Platinum-Based Chemotherapiess Block Growth of Ovarian Tumor Xenografts in vivo.*

Potential antitumor effects of the angiostatic steroid squalamine were assessed in murine tumor xenografts in the absence and presence of cisplatin or carboplatin chemotherapy. Human ovarian 2008 cancer cells without or with HER-2 overexpression were grown as subcutaneous tumors in nude mice. Tumors were grown to 150-200 mm^3 in size. Then, animals with established tumors were treated with control solution, cisplatin alone at two different dose levels (4 mg/kg on day 1 or 5 mg/kg on days 1,8), squalamine alone (2 mg/kg) on days 1-10, or cisplatin in combination with squalamine (days 1-10) (see Fig. 1 in ref. 27). In one set of experiments, animals were treated with a high dose of cisplatin near the maximum tolerated dose (5 mg/kg on days 1,8) (27). In the

second set of experiments, lower doses of cisplatin that resulted in only partial growth inhibition (26,29) were chosen in order to ensure use of the chemotherapeutic agant at a level that would not totally suppress tumor growth, thus allowing detection of any potential additive effects of a squalamine-cisplatin interaction. By 28 days, both 2008 parental and HER-2-overexpressing tumors showed little overall response to therapy with the lower dose of cisplatin alone. However, 2008 parental and HER-2-overexpressing tumors exhibited some minor response to cisplatin alone administered at the higher dose level (27). Squalamine elicited a partial reduction in tumor size as compared to controls in both 2008 parental and HER-2-overexpressing tumors. More profound tumor growth inhibition was elicited by combined treatment with squalamine and cisplatin in both 2008 parental and HER-2-overexpressing cancers (27). This effect of combination therapy was found when squalamine was administered with either low or high doses of cisplatin (27).

The antitumor effects of squalamine with and without platinum-based chemotherapy were also assessed using a different human ovarian tumor xenograft, CAOV3, that has been transfected to exhibit HER-2 over-expression (see Fig. 2A in ref. 27). After tumor growth to 50-60 mm^3, animals were treated with control solution, carboplatin alone (60 mg/kg) on day 1, squalamine alone (2 mg/kg) on days 1-10, or carboplatin (day 1) in combination with squalamine (days 1-10). By 28 days, CAOV3 HER-2-overexpressing tumors showed minimal response to therapy with carboplatin alone (27). As with the 2008 tumors, squalamine as a single agent elicited a partial reduction in CAOV3 tumor size as compared to controls (27). More marked inhibition of tumor growth was elicited by combined treatment with squalamine and carboplatin (27).

The tumor growth inhibition seen with combined squalamine and platinum-based chemotherapeutics for both human ovarian tumor lines persisted for up to 18 days following cessation of squalamine treatment. We therefore investigated how long bioactivity persisted with combined cisplatin and squalamine treatment of HER-2-overexpressing CAOV3 tumors by maintaining the dual therapy animal cohort until the mean tumor size for these animals reached 500 mm^3 (see Fig. 2B in ref. 27). After tumor growth to 50-60 mm^3, animals with established tumors were treated with control solution, cisplatin alone (4 mg/kg) on day 1, squalamine alone (2 mg/kg) on days 1-10, or cisplatin (day 1) combined with squalamine (days 1-10). As compared with control tumor xenografts, the calculated tumor growth delay in established tumors was 7 days for cisplatin therapy alone, 28 days for squalamine treatment alone, and 91 days for squalamine with cisplatin (27). Combined squalamine-cisplatin therapy was nontoxic as assayed by no animal death or significant weight loss during the study period (27).

3.b. *Squalamine and Cisplatin Promote Ovarian Tumor Cell Apoptosis in vivo.*

To assess molecular effects of squalamine and cisplatin, ovarian 2008 parent and HER-2-overexpressing tumor xenografts remaining after treatments with squalamine, cisplatin or a combination of the reagents were harvested and assessed for ovarian tumor cell apoptosis *in vivo*. For evaluation of apoptosis, the modified TUNEL assay (34, 35) was performed on tissue sections. The assays showed evidence of increased apoptosis in ovarian 2008 parental tumor cells treated with squalamine alone, cisplatin or combined cisplatin-squalamine as compared to appropriate controls (all at $P<0.05$) (see Fig. 3A in ref. 27). The 2008 HER-2-overexpressing ovarian tumors displayed less apoptotic activity than 2008 parental cancers with all treatments (27). Although apoptosis tended to be higher after administration of either squalamine or cisplatin alone, only treatment with squalamine in combination with cisplatin elicited a significant increase in the extent of apoptosis of HER-2-overexpressing ovarian cancers ($P<0.001$) (27). The results suggest that squalamine enhances cytotoxic effects of cisplatin chemotherapy for human ovarian cancer cells by increasing levels of tumor cell apoptosis produced by cisplatin exposure, either with or without HER-2 oncogene overexpression. In preliminary experiments, similar results have been obtained after treatments with squalamine in combination with carboplatin (data not shown).

3.c. *Squalamine Down-Regulates Ovarian Tumor-Associated Angiogenesis but not VEGF Production in vivo.*

Tissue sections of parent and HER-2-overexpressing 2008 tumor xenografts remaining after treatments with squalamine, cisplatin or a combination of the reagents were prepared for immunohistochemical staining with human von Willebrand Factor (vWF) to detect blood vessels (27,34). On scoring of tumor microvessel density, 2008 HER-2-overexpressing tumors exhibited more angiogenic activity than 2008 parental cancers ($P<0.001$) (see Fig. 3B in ref. 27). Treatment with squalamine alone elicited a reduction of tumor-associated blood vessel density for either ovarian tumor (27), and the immunohistochemical analyses also revealed a reduction of tumor-associated angiogenesis in mice treated with cisplatin plus squalamine ($P<0.01$) (27). No significant differences in microvessel density were found between groups treated with cisplatin alone and controls. The results suggest that squalamine is antiangiogenic for ovarian cancer cells with or without HER-2 overexpression. Squalamine-induced suppression of tumor microvessels is also a sustainable event since it was noted up to 18 days following the last squalamine dose. Similar studies based on treatments with squalamine combined with carboplatin are underway.

KEY RESEARCH ACCOMPLISHMENTS

- Profound growth inhibition was elicited by squalamine alone and by combined treatment with squalamine and cisplatin or squalamine and carboplatin for both parental and HER-2-overexpressing ovarian tumor xenografts.

- Immunohistochemical evaluation of tumors revealed decreased microvessel density and increased apoptosis. Although HER-2-overexpressing tumors had more angiogenic and less apoptotic activity than parental cancers, growth of both tumor types appear to be similarly suppressed by treatment with squalamine combined with cisplatin or squalamine combined with carboplatin.

- In *in vitro* studies, we found that squalamine does not directly affect proliferation of ovarian cells. However, squalamine significantly blocked VEGF-induced activation of p42/p44 MAP kinase and cell proliferation in human vascular endothelial cells.

- The results suggest that squalamine is anti-angiogenic for ovarian cancer xenografts and appears to enhance cytotoxic effects of cisplatin and carboplatin chemotherapy independent of HER-2 tumor status.

- It is important to note that this preclinical work has helped to promote the initiation of independent clinical trials of squalamine for treatment of patients with resistant or recurrent ovarian cancer (38).

REPORTABLE OUTCOMES

Presentations

1. Pietras, R.J. "Squalamine-induced blockade of tumor-associated angiogenesis in human ovarian cancers". Presented at Jonsson Comprehensive Cancer Center Lecture Series, UCLA (February, 2002).

2. Pietras, R.J. "New approaches to antitumor therapy". Presented at Marion Medical Center Cancer Forum, Santa Maria, California (September, 2002).

Publications

1. Li, D., Williams, J. I. and Pietras, R.J. (2002). Squalamine and cisplatin block angiogenesis and growth of human ovarian cancer cells with or without HER-2 gene overexpression. Oncogene **21** : 2805-2814.

Additional Research Opportunities

Results from the preclinical research activity outlined above has led to the promotion of new clinical trials of squalamine in the treatment of patients with resistant or recurrent ovarian cancer. The PI of the present grant, Dr. Pietras, was a co-investigator in this ongoing series of clinical trials:

1. Davidson, S. A., Chap, L., Pietras, R., Astrow, A., Gajewski, W., Brader, F., Petrone, M., Desai, A., Solomon, S., Holroyd, K., Major, F., Adler, L. and Cohn, A. (2002). A Phase IIA trial of continuous 5-day infusions of MSI-1256F (squalamine lactate) plus carboplatin for therapy of persistent or recurrent advanced ovarian cancer. Proc. Am. Soc. Clin. Oncol. : 878.

No patents, development of cell lines, informatics or additional funding opportunities to be reported at this time.

CONCLUSIONS

The potential role of squalamine, a natural anti-angiogenic sterol, in treatment of ovarian cancers with or without standard cisplatin or carboplatin chemotherapy was assessed. Since HER-2 gene overexpression is associated with cisplatin and carboplatin resistance *in vitro* and promotion of tumor angiogenesis *in vivo*, the response of ovarian cancer cells with or without HER-2 gene overexpression to squalamine and cisplatin or squalamine and carboplatin was also evaluated in tumor xenograft models and in tissue culture. Profound growth inhibition was elicited by squalamine alone and by combined treatment with squalamine and cisplatin or squalamine and carboplatin for both parental and HER-2-overexpressing ovarian tumor xenografts. Immuno-histochemical evaluation of tumors showed decreased microvessel density and increased apoptosis. Although HER-2-overexpressing tumors had more angiogenic and less apoptotic activity than parental cancers, growth of both tumor types was similarly suppressed by treatment with squalamine combined with cisplatin. In *in vitro* studies, we found that squalamine does not directly affect proliferation of ovarian cells. However, squalamine significantly blocked VEGF-induced activation of p24/p44 MAP kinase and cell proliferation in human vascular endothelial cells. The results suggest that squalamine is anti-angiogenic for ovarian cancer xenografts and appears to enhance cytotoxic effects of cisplatin and carboplatin chemotherapy independent of HER-2 tumor status. Further experiments are ongoing in accord with our statement of work. In addition, it is important to note that this preclinical work has helped to promote the initiation of clinical trials of squalamine for treatment of patients with resistant or recurrent ovarian cancer.

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A phase IIA trial of continuous 5-day infusions of MSI-1256F (squalamine lactate) plus carboplatin for therapy of persistant or recurrent advanced ovarian cancer

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Abstract: Squalamine (MSI-1256F) is an anti-angiogenic aminosterol that acts directly on activated endothelial cells after intracellular uptake. MSI-1256F when administered as a five-day continuous infusion in conjunction with carboplatin (AUC=6) every three weeks at 200mg/m2/day. Thirty three patients with stage III or stage IV ovarian cancer, with either progression on primary paclitaxel and carboplatin therapy (refractory disease) or recurrence within 6 months of initial response to paclitaxel and carboplatin therapy (resistant disease) received squalamine lactate. Patients with recurrence, resistant or sensitive, within 12 months of initial response to a secondary or tertiary regimen were also permitted (recurrent disease). PATIENT CHARACTERISTICS: median age 59 years (range 25-73 years), 28 Caucasian, 2 African American, 1 Asian, and 2 Hispanic, ECOG PS 0-1 (18 –0, 15-1), 21 stage III and 12 stage IV. Response data is available for 22 evaluable patients at this time. Median time on the study was 81 days. There were 8 objective responses in the first 22 evaluable patients. Survival data is maturing. TOXICITY: Grade 4 thrombocytopenia, anemia, leukopenia, myalgia and asthenia occurred in 3 patients. Other grade 4 adverse events like headache, nausea, pain and allergic reaction to carboplatin occurred in two patients. Also single incidence of anorexia, nausea, vomiting, diarrhea, constipation, atrial fibrillation, tachycardia, sinus bradycardia, hypokalemia, hyponatremia, dyspnea, increased cough, apnea, liver function abnormalities, injection site reaction and dizziness were reported. Additional patients are being enrolled at the 200 mg/m^2/day squalamine dose to complete enrollment in the study. Updated data on these additional patients and survival data will be presented. These results with Squalamine suggest it to be an exciting option for advanced refractory ovarian cancer patients, a group of patients for whom new therapeutic alternatives are much needed. Further studies are being planned.

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Squalamine and cisplatin block angiogenesis and growth of human ovarian cancer cells with or without HER-2 gene overexpression

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Angiogenesis is important for growth and progression of ovarian cancers. Squalamine is a natural antiangiogenic sterol, and its potential role in treatment of ovarian cancers with or without standard cisplatin chemotherapy was assessed. Since HER-2 gene overexpression is associated with cisplatin resistance *in vitro* and promotion of tumor angiogenesis *in vivo*, the response of ovarian cancer cells with or without HER-2 gene overexpression to squalamine and cisplatin was evaluated both in tumor xenograft models and in tissue culture. Ovarian cancer cells with or without HER-2 overexpression were grown as subcutaneous xenografts in nude mice. Animals were treated by intraperitoneal injection with control vehicle, cisplatin, squalamine or cisplatin combined with squalamine. At the end of the experiment, tumors were assessed for tumor growth inhibition and for changes in microvessel density and apoptosis. Additional *in vitro* studies evaluated effects of squalamine on tumor and endothelial cell growth and on signaling pathways in human endothelial cells. Profound growth inhibition was elicited by squalamine alone and by combined treatment with squalamine and cisplatin for both parental and HER-2-overexpressing ovarian tumor xenografts. Immunohistochemical evaluation of tumors revealed decreased microvessel density and increased apoptosis. Although HER-2-overexpressing tumors had more angiogenic and less apoptotic activity than parental cancers, growth of both tumor types was similarly suppressed by treatment with squalamine combined with cisplatin. In *in vitro* studies, we found that squalamine does not directly affect proliferation of ovarian cells. However, squalamine significantly blocked VEGF-induced activation of MAP kinase and cell proliferation in human vascular endothelial cells. The results suggest that squalamine is anti-angiogenic for ovarian cancer xenografts and appears to enhance cytotoxic effects of cisplatin chemotherapy independent of HER-2 tumor status.

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Introduction

Ovarian cancer is the most deadly gynecologic malignancy. About 26 500 women are diagnosed with this cancer each year and have an overall 5-year survival rate of only 47% (Ozols, 1999). For most patients, surgery alone does not cure the cancer due to the spread of tumors beyond the confines of the ovary, and management in the clinic often requires use of toxic chemotherapy regimens. The progressive growth and spread of ovarian cancer depends, in part, on the formation of an adequate blood supply, and tumor angiogenesis has been reported to have prognostic significance in epithelial ovarian cancer (Alvarez *et al.*, 1999). Therapy directed toward the vasculature of solid tumors is now being pursued as an important new direction in cancer treatment because avascular tumors exhibit only limited growth and tumor aggressiveness, and metastatic potential commonly correlates with tumor vascularity (Folkman, 1971; Gimbrone *et al.*, 1972).

Vascular endothelial growth factor (VEGF) is produced by most solid tumors and elicits a mitogenic effect on tumor-associated endothelial cells (Keck *et al.*, 1989; Leung *et al.*, 1989). VEGF binding to receptor tyrosine kinases triggers activation of downstream signaling enzymes, including MAP kinase, which, in turn, regulate gene expression and specific endothelial cell responses including proliferation, migration, differentiation and apoptosis (Soker *et al.*, 1996; Rousseau *et al.*, 1997). Several studies suggest that VEGF plays an important role in progression of ovarian cancer (Paley *et al.*, 1997; Yamamoto *et al.*, 1997; Alvarez *et al.*, 1999), and the ability of VEGF to increase vascular permeability (Ferrara *et al.*, 1993; Keck *et al.*, 1989; Leung *et al.*, 1989) may also promote formation of malignant ascites (Zebrowski *et al.*, 1999). Growth factor pathways, such as those dependent on the HER-2 receptor, appear to up-regulate VEGF production in some solid tumors (Petit *et al.*, 1997). Since HER-2 receptor is overexpressed in a significant number of ovarian cancers (Slamon *et al.*, 1989; Wong

et al., 1995; Hellstrom *et al.*, 2001), it may also play a role in promoting further growth of ovarian malignancy by increasing VEGF-dependent tumor angiogenesis.

Squalamine, a natural sterol from tissues of the dogfish shark (Moore *et al.*, 1993), has significant anti-angiogenic and antitumor activity in laboratory models of brain, breast and lung cancer (Sills *et al.*, 1998; Teicher *et al.*, 1998; Schiller and Bittner, 1999). Squalamine is a 7,24-dihydroxylated 24-sulfated cholestane steroid conjugated to spermidine at C-3. Squalamine blocks endothelial cell growth and has inhibitory activity in chick embryo chorioallantoic membrane and rabbit corneal micropocket assays (Sills *et al.*, 1998; Williams, 1999). This anti-angiogenic agent may have good potential for clinical application because it inhibits endothelial cell proliferation induced by a wide range of growth factors, including VEGF (Sills *et al.*, 1998). This inhibition may result, in part, from its interaction with endothelial cell surface proton pumps, thereby altering intracellular pH and impeding signaling by growth factors (Akhter *et al.*, 1999; Eckhardt, 1999). However, a receptor with high affinity for binding squalamine has not yet been identified. When administered as a single agent in nude mice with lung cancer xenografts, squalamine has limited antitumor activity, but the anti-angiogenic steroid significantly enhances the antitumor efficacy of cisplatin and carboplatin/paclitaxel chemotherapies (Teicher *et al.*, 1998; Schiller and Bittner, 1999; Williams, 1999). Since platinum-based treatments are often used for human ovarian cancers (Ozols, 1999), squalamine in combination with cisplatin was studied to assess its utility as part of a coordinated attack against human ovarian cancers and their blood supply. One additional feature we were particularly interested in was the consequence of HER-2 oncogene overexpression for squalamine modulation of growth in ovarian tumor xenografts. Amplification and/or overexpression of HER-2 proto-oncogene in human cancers, including ovarian cancers, is often associated with poor clinical outcome (Slamon *et al.*, 1989; Wong *et al.*, 1995), and human ovarian tumor cells with overexpression of HER-2 membrane receptor also exhibit resistance to cisplatin (Pegram *et al.*, 1997). We therefore examined whether the level of HER-2 expression in paired HER-2-transfected and non-transfected ovarian cancers influenced the degree of tumor growth inhibition seen with squalamine with or without concomitant platinum-based treatment.

Results

Squalamine and platinum-based chemotherapeutics block growth of ovarian tumor xenografts in vivo

Potential antitumor effects of the angiostatic steroid squalamine were assessed in murine tumor xenografts in the absence and presence of cisplatin chemotherapy. Human ovarian 2008 cancer cells without (Figure 1a) or with HER-2 overexpression (Figure 1b) were grown as subcutaneous tumors in nude mice. Tumors were grown to 150–200 mm^3 in size. Then, animals with established tumors were treated with control solution, cisplatin alone, squalamine alone (2 mg/kg) on days 1–10, or cisplatin in combination with squalamine (days 1–10).

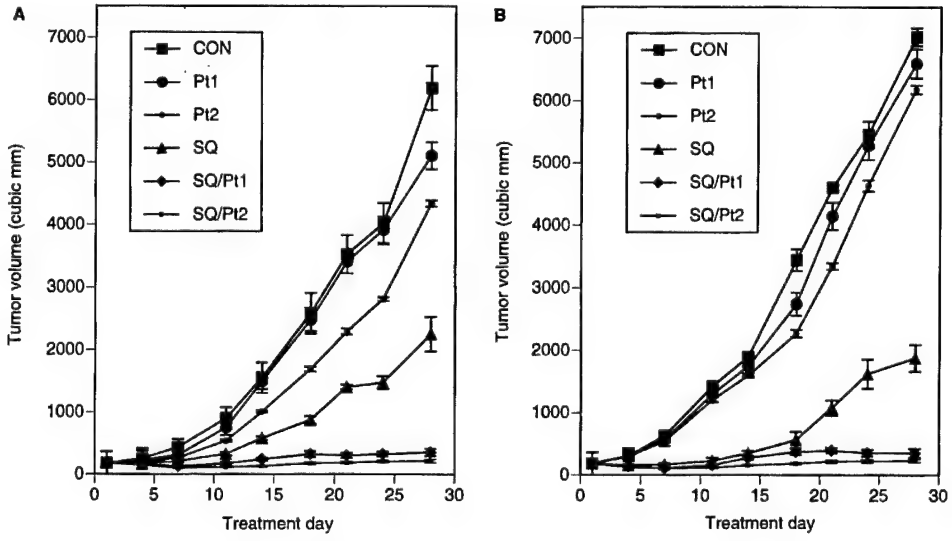

Figure 1 Squalamine inhibits growth of ovarian 2008 parental and HER-2-overexpressing tumor xenografts in nude mice and enhances the cytotoxic effects of cisplatin. (**a**) Ovarian 2008 parental tumor cells were inoculated subcutaneously in nude mice. (**b**) Ovarian 2008 HER-2-overexpressing cancer cells were inoculated subcutaneously in nude mice. After 7 days, animals with tumors of comparable size were randomized to treatment with control vehicle (CON), squalamine (SQ; 2 mg/kg on days 1–10), cisplatin at two different doses (Pt 1 = 4 mg/kg on day 1; Pt 2 = 5 mg/kg on days 1, 8), or cisplatin administered in combination with squalamine (SQ/Pt 1; or SQ/Pt 2). At the end of the experiment, HER-2 receptor expression levels were assessed and, as possible, confirmed to be low in parental tumors and high in HER-2-overexpressing xenografts. Results are expressed as mean ± s.e.m. for tumor volumes (mm^3) measured over a 28-day experimental period

Cisplatin was administered at two different dose levels (see Pietras *et al*., 1994; Pegram *et al*., 1997). In one set of experiments, animals were treated with a high dose of cisplatin near the maximum tolerated dose (5 mg/kg on day 1 and day 8; Pt 2). In the second set of experiments, lower doses of cisplatin that resulted in only partial growth inhibition (4 mg/kg on day 1; Pt 1) were chosen in order to ensure use of the chemotherapeutic agent at a level that would not totally suppress tumor growth, thus allowing detection of any potential additive effects of a squalamine-cisplatin interaction. By 28 days, both 2008 parental (Figure 1a) and HER-2-overexpressing (Figure 1b) tumors showed little overall response to therapy with the lower dose of cisplatin alone (both at $P>0.05$). However, 2008 parental ($P<0.01$) and HER-2-overexpressing ($P<0.05$) tumors exhibited some minor responses to cisplatin administered at high doses (see Figure 1). Squalamine elicited a partial reduction in tumor size as compared to controls ($P<0.01$) in both 2008 parental (Figure 1a) and HER-2-overexpressing (Figure 1b) tumors. More profound tumor growth inhibition (94–95% of controls) was elicited by combined treatment with squalamine and cisplatin ($P<0.001$) in both 2008 parental (Figure 1a) and HER-2-overexpressing (Figure 1b) cancers. This effect of combination therapy was found when squalamine was administered with either low or high doses of cisplatin.

The antitumor effects of squalamine with and without platinum-based chemotherapy were also assessed using a different ovarian tumor xenograft, CAOV3, that has been transfected to exhibit HER-2 overexpression (Figure 2a). After tumor growth to 50–60 mm^3, animals were treated with control solution, carboplatin alone (60 mg/kg) on day 1, squalamine alone (2 mg/kg) on days 1–10, or carboplatin (day 1) in combination with squalamine (days 1–10). By 28 days, CAOV3 HER-2-overexpressing tumors showed minimal response to therapy with carboplatin at a dose below the maximum tolerated dose (Figure 2a). As with the 2008 tumors, squalamine as a single agent elicited a partial reduction in CAOV3 tumor size as compared to controls ($P<0.05$) (Figure 2a). More marked inhibition of tumor growth was elicited by combined treatment with squalamine and carboplatin ($P<0.001$; Figure 2a).

The tumor growth inhibition seen with combined squalamine and platinum-based chemotherapeutics for both human ovarian tumor lines persisted for up to 18 days following cessation of squalamine treatment. We therefore investigated how long bioactivity persisted with combined cisplatin and squalamine treatment of HER-2-overexpressing CAOV3 tumors by maintaining the dual therapy animal cohort until the mean tumor size for these animals reached 500 mm^3 (Figure 2b). After tumor growth to 50–60 mm^3, animals with established tumors were treated with control solution, cisplatin alone (4 mg/kg) on day 1, squalamine alone (2 mg/kg) on days 1–10, or cisplatin (day 1) in combination with squalamine (days 1–10). As compared with control tumor xenografts, the calculated tumor growth delay in established tumors was 7 days for cisplatin therapy alone, 28 days for squalamine treatment alone, and 91 days for squalamine combined with cisplatin administration (Figure 2b). Combined squalamine-cisplatin therapy was nontoxic as assayed by no animal death or significant weight loss during the study period.

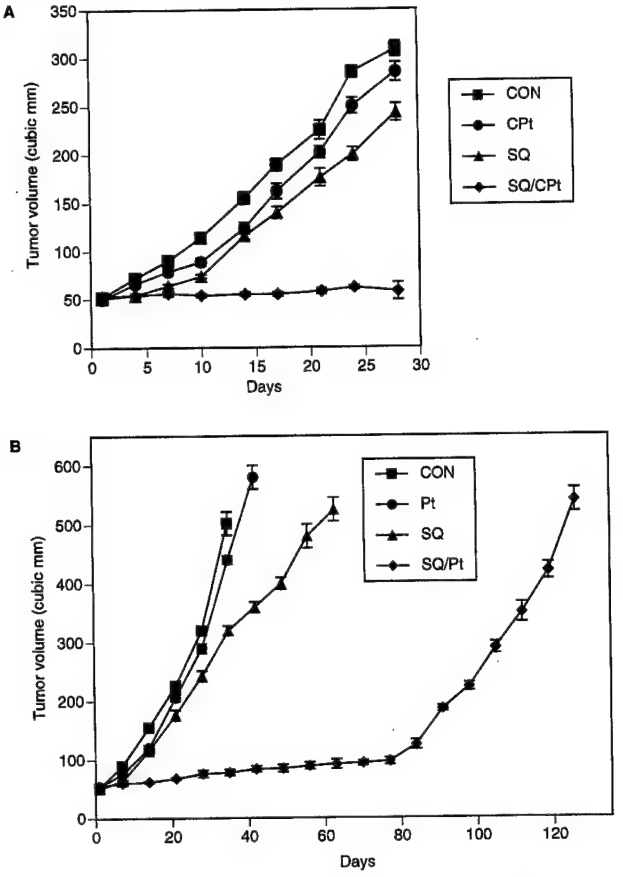

Figure 2 Squalamine inhibits growth of ovarian CAOV3 tumor xenografts with HER-2 overexpression and enhances the cytotoxic effects of cisplatin and carboplatin. (**a**) CAOV3 ovarian cancer cells were inoculated subcutaneously in nude mice. After 7 days, animals with tumors of comparable size were randomized to treatment with control solution (CON), squalamine (SQ; 2 mg/kg on days 1–10), carboplatin (CPt; 60 mg/kg on day 1), or carboplatin given in combination with squalamine (SQ/CPt). At the end of the experiment, HER-2 receptor expression levels were assessed and, as possible, confirmed to be low in parental tumors and high in HER-2-overexpressing xenografts. Results are given as mean±s.e.m. for tumor volumes (mm^3) measured over a 28-day experimental period. (**b**) CAOV3 ovarian cancer cells were inoculated subcutaneously in nude mice. After 7 days, animals with tumors of comparable size were randomized to treatment with control solution (CON), squalamine (SQ; 2 mg/kg on days 1–10), cisplatin (Pt; 4 mg/kg on day 1), or cisplatin administered in combination with squalamine (SQ/Pt). Tumor volumes (mm^3) are expressed as mean±s.e.m. for measurements to assess tumor growth delay due to the several treatments (Teicher *et al*., 1998)

Squalamine and cisplatin promote ovarian tumor cell apoptosis in vivo

To assess molecular effects of squalamine and cisplatin, ovarian 2008 parent and HER-2-overexpressing tumor

xenografts remaining after treatments with squalamine, cisplatin (4 mg/kg dose; Pt 1) or a combination of the reagents were harvested at day 28 and assessed for ovarian tumor cell apoptosis *in vivo*. For evaluation of apoptosis, the modified TUNEL assay (Ellis *et al.*, 1991; Steller, 1995) was performed on tissue sections. The assays showed evidence of increased apoptosis in ovarian 2008 parental tumor cells treated with squalamine alone, cisplatin or combined cisplatin-squalamine as compared to appropriate controls (all at $P<0.05$) (Figure 3a). The 2008 HER-2-overexpressing ovarian tumors displayed less apoptotic activity than 2008 parental cancers with all treatments ($P<0.05$). Although apoptosis tended to be higher after administration of either squalamine or cisplatin alone, only treatment with squalamine in combination with cisplatin elicited a significant increase in the extent of apoptosis of HER-2-overexpressing ovarian cancers ($P<0.001$) (Figure 3a). The results suggest that squalamine enhances cytotoxic effects of cisplatin chemotherapy for human ovarian cancer cells by increasing levels of tumor cell apoptosis produced by cisplatin exposure, either with or without HER-2 oncogene overexpression.

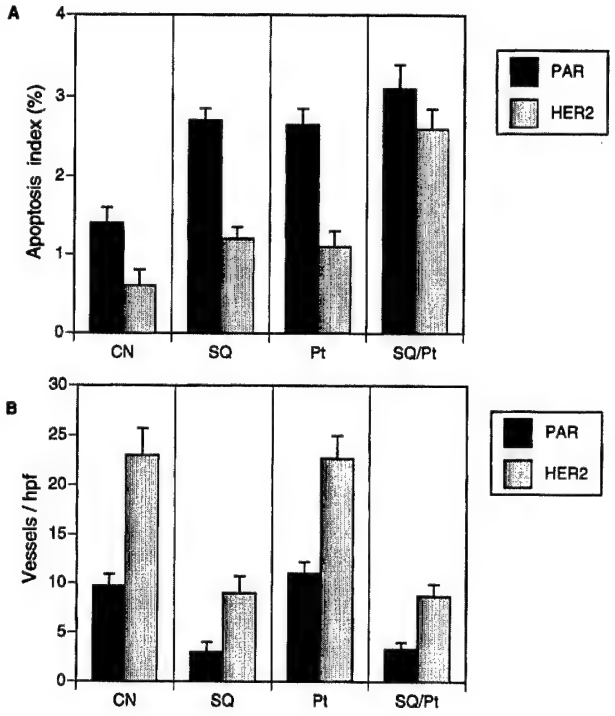

Figure 3 Effects of squalamine and cisplatin on ovarian tumor cell apoptosis and tumor-associated angiogenesis *in vivo*. (**a**) A modified TUNEL assay was performed in ovarian 2008 parental (PAR) and HER-2-overexpressing (HER2) tumor tissue sections to compare tumor cell apoptosis *in vivo* among the different treatment groups, including control (CN), squalamine (SQ; 2 mg/kg on days 1–10), cisplatin (Pt 1; 4 mg/kg on day 1), and squalamine-cisplatin (SQ/Pt 1; squalamine in combination with cisplatin) Figure 1.). The apoptotic index was assessed as before (O'Reilly *et al.*, 1997). (**b**) Squalamine and cisplatin inhibit tumor-associated angiogenesis in ovarian 2008 parental and HER-2-overexpressing tumor xenografts in nude mice. Immunohistochemical staining for von Willebrand Factor was used to quantitate blood vessel density (number of blood vessels per high power field) in tumor sections. The several treatment groups included control (CN), squalamine (SQ; 2 mg/kg on days 1–10), cisplatin (Pt 1; 4 mg/kg on day 1), and squalamine-cisplatin (SQ/Pt 1; squalamine in combination with cisplatin) (see Figure 1a,b). Results are expressed as mean ± s.e.m. of appropriate values

Squalamine down-regulates ovarian tumor-associated angiogenesis but not VEGF production in vivo

Tissue sections of parent and HER-2-overexpressing 2008 tumor xenografts remaining after treatments with squalamine, cisplatin (4 mg/kg dose; Pt 1) or a combination of the reagents at day 28 (Figure 1) were also prepared for immunohistochemical staining with human von Willebrand Factor (vWF) to detect blood vessels. On review and scoring of tumor microvessel density, 2008 HER-2-overexpressing tumors exhibited more angiogenic activity than 2008 parental cancers ($P<0.001$) (Figure 3b). Treatment with squalamine alone elicited a significant reduction of tumor-associated blood microvessel density for either ovarian tumor ($P<0.001$) (Figure 3b), and the immunohistochemical analyses also revealed a reduction of tumor-associated angiogenesis in mice treated with cisplatin plus squalamine ($P<0.01$) (Figure 3b). No significant differences in microvessel density were found between groups treated with cisplatin alone and controls. The results suggest that squalamine is anti-angiogenic for ovarian cancer cells with or without HER-2 overexpression. Squalamine-induced suppression of tumor microvessels is also a sustainable event since it was noted up to 18 days following the last squalamine dose.

In further studies on expression of VEGF in tumor xenografts *in vivo*, ovarian 2008 parent and HER-2-overexpressing tumors were freshly excised at the end of the experiment (day 28) and dissected free of contaminating mouse tissue. The tumor xenografts were then dissociated and homogenized for further analysis of VEGF expression by Western blot (Figure 4). Under nonreducing conditions, protein bands of the appropriate molecular size occur in both 2008 parent and HER-2-overexpressing tumors (Ferrara *et al.*, 1993). Moreover, 2008 HER-2-overexpressing tumors appear to have significantly higher levels of VEGF than the 2008 parent tumors (Figure 4). Among parental and HER-2-overexpressing tumors treated *in vivo* with squalamine, levels of VEGF were comparable to those of appropriate paired controls (Figure 4), suggesting that squalamine treatment does not influence tumor-induced VEGF production.

Squalamine blocks VEGF-stimulated proliferation of endothelial cells in vitro

To assess the biologic mechanism for the anti-angiogenic and antitumor effects of squalamine noted with the ovarian 2008 parent and 2008 HER-2-overexpressing tumor xenografts, human

umbilical vein endothelial cells (HUVEC) were grown *in vitro*. VEGF alone elicits significant proliferation of HUVEC cells by 72 h. In the absence of VEGF, squalamine had no effect on survival or proliferation of the endothelial cells. However, in the presence of VEGF, squalamine elicited a significant reduction in VEGF-induced endothelial cell proliferation ($P<0.001$) (Figure 5). This growth-suppressive effect of squalamine appears restricted to endothelial cells since the compound had no direct inhibitory effect on the proliferation of ovarian 2008 cancer cells, either with or without HER-2 gene overexpression (Figure 5).

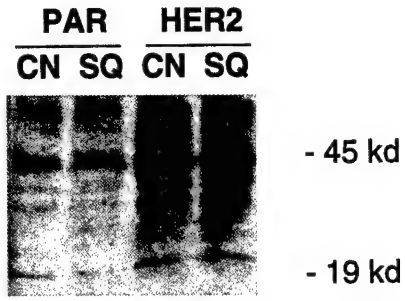

Figure 4 Higher levels of VEGF protein in ovarian HER-2-overexpressing tumors as compared with that of parental tumors grown *in vivo*. Ovarian 2008 parental (PAR) and HER-2-overexpressing (HER2) tumor xenografts treated *in vivo* without squalamine (CN) or with squalamine (SQ) were freshly harvested, dissociated, homogenized and processed for electrophoresis and Western blotting with anti-VEGF antibody. Equal amounts of protein were loaded in each lane. $VEGF_{165}$ is known to be the most abundant molecular species in the majority of cells, and purified $VEGF_{165}$ is about 45 kd in size under nondenaturing conditions, but the molecule also occurs in monomeric forms ranging from 18–23 kd depending, in part, on glycosylation (Ferrara *et al*., 1993)

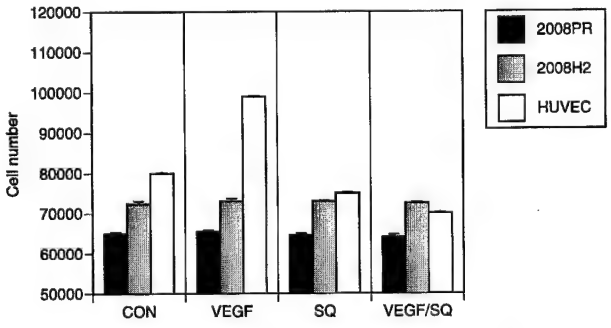

Figure 5 Squalamine inhibits VEGF-induced proliferation of endothelial cells *in vitro*, but does not directly affect the growth of ovarian epithelial tumor cells *in vitro*. Human umbilical vein endothelial cells (HUVEC), ovarian 2008 parental cells (2008PR) and ovarian 2008 HER-2-overexpressing cells (2008H2) were grown *in vitro* in the presence of vascular endothelial cell growth factor (VEGF; 20 ng/ml) or control vehicle (CON), squalamine (SQ; 16 μM) or combinations of VEGF with squalamine (VEGF/SQ) for three days. In additional control studies, squalamine at 8 and 16 μM also elicited no direct effect on proliferation *in vitro* of CAOV3 ovarian cancer cells with HER-2 overexpression (data not shown). All data are from triplicate determinations of cell numbers, with results presented as mean $\pm$ s.e.m.

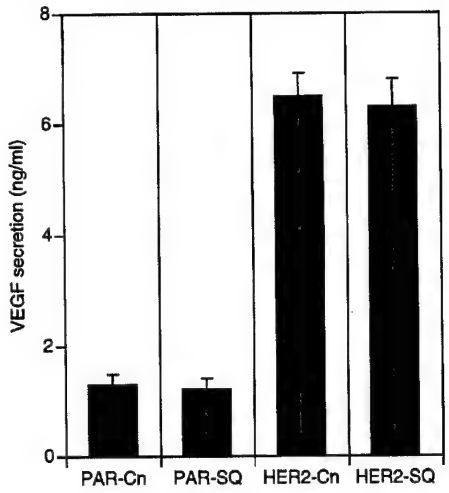

Figure 6 VEGF secretion by ovarian cancer cells with or without HER-2 gene overexpression. Ovarian 2008 parental cells (PAR) and ovarian 2008 HER-2-overexpressing cells (HER2) were grown *in vitro*, either with control medium (Cn) or medium containing squalamine (SQ; 16 μM). After 72 h, media were collected and processed for ELISA assay of VEGF secretion. Using a lower dose of squalamine at 8 μM for treatment of ovarian 2008 cells with HER-2 overexpression, similar effects on VEGF secretion were found ($n=2$; data not shown). Data represent mean $\pm$ s.e.m. units of VEGF secretion

Squalamine does not affect VEGF secretion in vitro *for ovarian cancer cells with or without HER-2 gene overexpression*

HER-2 overexpression is generally thought to lead to tumor development through its effects on promoting uncontrolled cancer cell growth. However, recent findings suggest that HER-2 may also regulate cell survival functions such as angiogenesis by promoting tumor production of VEGF (Petit *et al*., 1997). To explore how HER-2 may contribute to angiogenesis in ovarian cancers, we evaluated HER-2 effects on *in vitro* VEGF secretion by human ovarian tumor cells. Parent and HER-2-overexpressing ovarian 2008 cancer cells were incubated for 72 h *in vitro*, and secretion of VEGF into conditioned media was determined by use of established enzyme-linked immunosorbant assay (ELISA) (Ferrara *et al*., 1993; Petit *et al*., 1997) (Figure 6). Parent ovarian cancer cells show significant secretion of VEGF, and, after transfection of ovarian cells with HER-2 gene to high levels, a further increase in the level of VEGF secretion was found, a result consistent with the *in vivo* findings described above (see Figure 4). In parallel *in vitro* studies, treatment of ovarian cancer cells with squalamine elicited no significant effect on secretion of VEGF (Figure 6), again reminiscent of our *in vivo* findings. Thus, HER-2 overexpression may contribute to angiogenesis through up-regulation of VEGF secretion in ovarian cancer, but squalamine is not anti-angiogenic at this step in tumor-associated angiogenesis since it does not appear to directly affect secretion of VEGF by ovarian epithelial tumor cells.

Squalamine blocks VEGF-induced activation of MAP kinase in vitro

VEGF exerts its biologic effects by binding with receptor tyrosine kinases, notably Flt-1 and Flk-1/KDR, present at the surface of endothelial cells (Mustonen and Alitalo, 1995; Soker *et al*., 1996). Post-receptor signal transduction regulates the effects of VEGF, and the proliferative action of VEGF in endothelial cells has been associated with VEGF-induced tyrosine phosphorylation and stimulation of mitogen-activated protein kinases (MAP kinase), extracellular signal-regulated kinase ERK-1 (p44 ^{MAPK}) and ERK-2 (p42 ^{MAPK}) (Soker *et al*., 1996; Rousseau *et al*., 1997). On the assumption that blockade of endothelial cell proliferation by squalamine may occur, in part, by suppression of MAP kinase signaling cascades induced by growth factors, VEGF-induced tyrosine phosphorylation of MAP kinases was assessed. As expected, VEGF promotes tyrosine phosphorylation of MAP kinase isoforms, with maximal effects evident by 10 min (Figure 7). However, after administration of squalamine, the VEGF-stimulated phosphorylation of MAP kinase isoforms is significantly suppressed, especially after 30 min exposure to VEGF (Figure 7).

Discussion

It is well-known that solid tumors are angiogenesis-dependent for growth, and angiogenesis is stimulated by tumor-secreted angiogenic factors, such as VEGF, that bind to tyrosine kinase receptors and promote endothelial cell proliferation and new capillary formation (Folkman, 1971; Gimbrone *et al*., 1972; Folkman and Haudenschild, 1980). Similarly, the present studies suggest that angiogenesis is essential for growth of ovarian tumors *in vivo*. Squalamine, a natural anti-angiogenic steroid, blocks VEGF-induced growth of human umbilical vein endothelial cells, suggesting it presumptively could have a positive role in regulating the growth of ovarian cancers. The experiments described in this paper show further that squalamine suppresses *in vivo* growth of ovarian cancer xenografts to a similar extent with or without HER-2 oncogene overexpression in the tumor and enhances the cytotoxic effects of platinum-based chemotherapy. Resistance to cisplatin treatment in ovarian tumors with or without HER-2-overexpression may therefore be overcome when the cisplatin is combined with squalamine, a feature of squalamine that could have direct application to the design of appropriate clinical trials.

Several studies suggest that VEGF plays an important role in the progression of ovarian cancer (Paley *et al*., 1997; Yamamoto *et al*., 1997; Alvarez *et al*., 1999), and evidence for interactions between VEGF-induced angiogenesis and growth factor receptor pathways in tumors is beginning to emerge. Since the HER-2 growth factor receptor is overexpressed in a significant number of ovarian cancers, it may play an important role in promoting growth of ovarian cancers (Slamon *et al*., 1989; Wong *et al*., 1995; Juhl *et al*., 1997; Hellstrom *et al*., 2001). In the laboratory, HER-2 amplification is rate-limiting for expression of the malignant phenotype of ovarian cancer in a dose-dependent manner (Juhl *et al*., 1997), and HER-2 antireceptor antibodies elicit cytostatic growth inhibition of ovarian cancer cells overexpressing HER-2 (Pietras *et al*., 1994). We have demonstrated in this paper that VEGF is produced by transfected and non-transfected human ovarian 2008 cancers, and stimulation of the HER-2 pathway appears to further up-regulate tumor secretion of VEGF. In our studies on VEGF expression in ovarian 2008 tumor xenografts *in vivo*, ovarian 2008 tumors overexpressing HER-2 were associated with significantly higher levels of VEGF than ovarian 2008 parent control tumors, consistent with data from the *in vitro* experiments with the same cancer cells. These findings correspond well with prior observations on the stimulatory influence of HER-2 signaling on VEGF production in human breast cancers (Petit *et al*., 1997). The data also are consistent with clinical data demonstrating HER-2 oncogene overexpression is associated with a poor prognosis for ovarian cancer patients with this abnormality (Slamon *et al*., 1989; Berchuck *et al*., 1990; Wong *et al*., 1995). In these individuals, VEGF overproduction may stimulate angiogenesis to a greater extent than would otherwise be seen, leading to rapid tumor growth and shorter patient survival times.

Squalamine specifically inhibits endothelial cell proliferation induced by a wide range of growth factors, including VEGF. This inhibition may result from its interaction with endothelial cell surface proton pumps, thus altering intracellular pH and impeding downstream signaling by growth factors (Akhter *et al*., 1999; Eckhardt, 1999). Alternatively, calcium-dependent cell signaling following exposure to growth factors

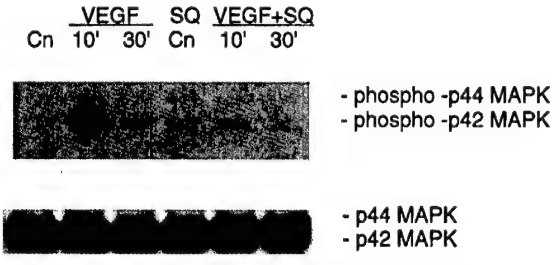

Figure 7 Squalamine blocks VEGF-stimulated tyrosine phosphorylation of MAP kinase in HUVEC cells *in vitro*. Quiescent HUVEC cells were treated with control vehicle (Cn), VEGF (50 ng/ml), squalamine (SQ; 1.6 μM) or VEGF in combination with squalamine (VEGF+SQ) for 10 or 30 min *in vitro*. Lysates were prepared and processed as described in Materials and methods. (**a**) Western blotting was performed with anti-phospho-p44/42 MAP kinase monoclonal antibody as indicated in Materials and methods. (**b**) Paired samples were used for Western blotting with anti-p44/42 MAP kinase monoclonal antibody to confirm similar total protein load in each lane. Treatment of HUVEC cells *in vitro* with squalamine at 0.8 μM elicited a similar block of VEGF-stimulated tyrosine phosphorylation of MAP kinase (data not shown)

such as VEGF may be dysregulated when squalamine forces intracellular redistribution of calmodulin (Chen *et al*., 1999). Irrespective of which mechanism may be operating, we find that squalamine blocks proliferation of human umbilical vein endothelial cells *in vitro*, while it does not directly interfere with growth of ovarian tumor cells or production of VEGF by these tumor cells *in vitro*. VEGF has been shown to initiate biologic responses by binding with receptor tyrosine kinases, including Flt-1, Flk-1/KDR and neuropilin, present at the surface of endothelial cells (Mustonen and Alitalo, 1995; Soker *et al*., 1998; Abedi and Zachary, 1997). The proliferative action of VEGF in endothelial cells is associated with the subsequent VEGF-induced tyrosine phosphorylation and stimulation in concert of focal adhesion kinase (FAK) and MAP kinases, including p38 MAP kinase, ERK-1 (p44 ^{MAPK}) and ERK-2 (p42 ^{MAPK}) (Soker *et al*., 1996; Rousseau *et al*., 1997; Kroll and Waltenberger, 1997). On testing the assumption that blockade of endothelial cell proliferation by squalamine may occur, in part, by suppression of MAP kinase signaling cascades induced by VEGF in endothelial cells, we found that squalamine significantly curbs VEGF-stimulated phosphorylation of MAP kinase isoforms p42 and p44 in HUVEC. Thus, squalamine may prevent endothelial cell growth and associated angiogenesis by interrupting signal transduction necessary for endothelial cell activation. In addition, administration of squalamine alone to nude mice with human ovarian cancer xenografts elicits a partial reduction in tumor size as compared to controls in both parental and HER-2-overexpressing tumors. This *in vivo* effect of squalamine is further enhanced by coadministration of cisplatin and is associated with a reduction of tumor-associated angiogenesis and with enhanced apoptosis of ovarian cancer cells with both 2008 tumor lines, notably when squalamine and cisplatin are used together. The chemotherapeutic effect on apoptosis presumably is due to interruption of the tumor-associated blood supply. Given the relatively small response of both ovarian tumor lines to cisplatin treatment, it is noteworthy that cisplatin plus squalamine is a far better treatment than squalamine alone, and the combination has similar antitumor efficacy with both tumor lines.

As in the experiments described above with ovarian cancers, squalamine alone was previously reported to be a potent inhibitor of the growth of blood vessel cells in preclinical studies of lung (Teicher *et al*., 1998; Schiller and Bittner, 1999) and brain (Sills *et al*., 1998) cancers. To evaluate the possible clinical efficacy of squalamine combined with standard cytotoxic antitumor therapy in suppressing growth of ovarian cancers, the anti-angiogenic sterol was administered to the 2008 parent and 2008 HER-2-overexpressing tumor xenografts in combination with cisplatin and to CAOV3 HER-2-overexpressing tumors in combination with cisplatin or carboplatin chemotherapy. Use of postoperative chemotherapy is standard treatment for all advanced-stage and for many early-stage patients with ovarian cancer, and adjuvant therapy with platinum-based regimens significantly prolongs survival (Ozols, 1999). The experimental results provide evidence of significant antitumor efficacy of the several ovarian cancers following combined treatment with squalamine and cisplatin (or carboplatin) as compared with appropriate controls. These results are consistent with earlier studies demonstrating enhancement by squalamine of the antitumor efficacy of cisplatin, carboplatin or carboplatin/paclitaxel therapies in nude mice with lung cancer xenografts (Teicher *et al*., 1998; Schiller and Bittner, 1999). Data in this paper show a strong correlation between the ability of squalamine-based chemotherapy to inhibit tumor angiogenesis and tumor growth and increase tumor apoptosis. Since platinum-based chemotherapies are often used for treatment of ovarian cancers (Ozols, 1999), extrapolation of our experimental results to the clinical setting leads us to infer squalamine in combination with platinum compounds may prove useful as part of a coordinated attack against both ovarian tumor blood supply and ovarian cancers.

Squalamine has been tested in two phase I clinical trials employing patients with a variety of solid malignancies and who have failed conventional therapies, and the chemical has been shown to be well-tolerated by human subjects (Patnaik *et al*., 1999). Phase II combination chemotherapy studies incorporating squalamine are ongoing in patients with advanced ovarian cancer. The outcome of these clinical trials will provide the first answers as to whether or not squalamine offers a degree of outcome improvement over cytotoxic therapy alone to patients afflicted with disseminated ovarian cancer comparable to that seen with the xenograft models described in this report.

Materials and methods

Cell lines

Human ovarian 2008 parental cells (provided by Dr S Howell) were derived from a patient with serous cystadenocarcinoma of the ovary (Disaia *et al*., 1972), and human CAOV3 ovarian carcinoma cells were obtained from American Type Culture Collection (Rockville, MD, USA). Using methods described elsewhere (Chazin *et al*., 1992; Pietras *et al*., 1994; Pegram *et al*., 1997), ovarian cancer cells with low expression of HER-2 gene were stably transfected with a vector containing the full-length cDNA of human HER-2 gene, with molecular characterization of HER-2 overexpression as before (Pietras *et al*., 1994; Pegram *et al*., 1997). Both parental and HER-2-overexpressing ovarian cancer cells were routinely cultured in RPMI 1640 media supplemented with 10% heat-inactivated fetal bovine serum, 2 mM freshly added glutamine and 1% penicillin G-streptomycin-fungizone solution (Irvine Scientific, Santa Ana, CA, USA). Human umbilical vein endothelial cells were obtained from Clonetics (San Diego, CA, USA), with immunospecific staining to confirm that cells are endothelial (Morales *et al*., 1995). The latter cells were grown in endothelial cell growth medium (EGM) which contains endothelial cell basal medium supplemented with 2% fetal bovine serum, 10 ng/ml hEGF, 1.0 μg/ml hydrocortisone,

50 μg/ml gentamicin, 50 ng/ml amphotericin-B, and 12 μg/ml bovine brain extract (BBE) (Folkman and Haudenschild, 1980; Morales *et al.*, 1995).

Measurement of VEGF protein levels

Secretion of VEGF protein was quantitated in extracellular media by enzyme-linked immunosorbant assay (ELISA) after centrifugation and concentration by Amicon® filtration (Ferrara *et al.*, 1993; Goldman *et al.*, 1993).

Quantitation of cell proliferation in vitro

Human ovarian tumor or endothelial cells (10 000 cells/well) were plated in 12-well plates and allowed to attach overnight in the appropriate complete media. The medium was then removed and replaced with either fresh complete medium, medium supplemented with squalamine, VEGF (PeproTech, Rocky Hill, NJ, USA), or squalamine plus VEGF. The choice of squalamine doses for use in the *in vitro* studies was based on results from preliminary experiments and pharmacokinetic studies (Williams *et al.*, 2001). Cell proliferation was determined by both direct counts and Coulter-counter analyses. All assays were performed in triplicate.

Detection of p44/42 MAP kinase activity by Western blot

Tyrosine phosphorylation of MAP kinase was determined as before (Sebolt-Leopold *et al.*, 1999). HUVEC cells were grown in 100 mm culture dishes to 80–90% confluence and in complete EGM media. Then, cells were starved in endothelial cell basal medium containing 1% fatty acid-free, steroid-depleted bovine serum albumin for 8 h. Prior to addition of VEGF (50 ng/ml) for various times at 37°C, cells were incubated in the presence or absence of 1.6 μM squalamine for 1 h. After treatment, cells were immediately rinsed with cold PBS three times and chilled at 0–4°C prior to lysis in 100 μl cold lysis buffer containing 20 mM Tris (pH 8.0), 137 mM NaCl, 10% glycerol, 1% Triton X-100, 20 mM EDTA, 0.5 μg/ml leupeptin, 0.5 μg/ml aprotinin, 50 μg/ml trypsin inhibitor, 0.1 mg/ml 4-(2-aminoethyl)-benzenesulfonyl fluoride and 1.0 mM sodium orthovanadate (Sigma, St. Louis, MO, USA). Total protein concentration was determined by BCA assay (Pierce Biochemical, Rockford, IL, USA). Protein samples (50 μg/lane) were separated on a 4–12% precast Tris-Glycine gel (Novex, San Diego, CA, USA). Proteins were then transferred to a nitrocellulose membrane and subjected to immunodetection with anti-phospho-p44/p42 MAP kinase E10 monoclonal antibody (New England Biolabs, Beverly, MA, USA), using the ECL Western blotting system (Amersham Pharmacia, Arlington Heights, IL, USA) as before (Chazin *et al.*, 1992; Pegram *et al.*, 1997). In paired control experiments, a p44/p42 MAP kinase antibody (New England Biolabs) was used for detection of total enzyme protein.

Tumor cell inoculation and drug treatment in nude mice

All animal studies were conducted according to protocols approved by the UCLA animal research committee. Five to six-week-old female athymic nude mice (25–30 gm) were obtained from Harlan Sprague-Dawley (Indianapolis, IN, USA) and primed for 7 days with 17β-estradiol applied subcutaneously in a biodegradable carrier-binder (1.7 mg of estradiol per pellet, 60-day release form, Innovative Research of America, Sarasota, FL, USA). Thereafter, 2×10^7 tumor cells were injected subcutaneously in the mid-back region of the mice and grown as xenografts (Pietras *et al.*, 1994; Pegram *et al.*, 1997). After growth of tumors to 150–200 mm^3, animals were randomized by body weight and tumor nodule size to different treatment groups of 5–7 mice. Animals were treated by intraperitoneal injection with control solution, cisplatin (Platinol), carboplatin (Paraplatin; Bristol Myers Squibb, Princeton, NJ, USA), squalamine (2 mg/kg on days 1–10; Genaera Corporation, Plymouth Meeting, PA, USA) or cisplatin (or carboplatin) in combination with squalamine. Based on previous work (Pietras *et al.*, 1994; Pegram *et al.*, 1997; Schiller and Bittner, 1999; Plumb *et al.*, 2000), cisplatin (4 mg/kg) and carboplatin (60 mg/kg) were generally administered at doses less than the maximum tolerated dose in an attempt to allow any additive effects of combination treatment with platinum-based agents and squalamine to be more easily detected (Schiller and Bittner, 1999). However, in selected experiments, cisplatin (5 mg/kg on day 1, 8) was also administered alone and in combination with squalamine at maximum tolerated doses as determined previously for these cell lines in nude mouse models (Pietras *et al.*, 1994; Pegram *et al.*, 1997; Kolfschoten *et al.*, 2000). The 2 mg/kg squalamine dose was selected as being the minimal daily dose previously shown to be significantly active in a tumor xenograft model in combination with a platinum agent (Williams *et al.*, 2001). Tumor nodules were monitored by dimension measurements as before (Pietras *et al.*, 1994). In most studies, experiments were terminated after 28 days to allow harvesting of tumor xenografts for assessment of selected molecular endpoints, including HER-2 expression by immunohistochemical methods (see Pietras *et al.*, 1994; Pegram *et al.*, 1997). Mean tumor growth inhibition for each treatment group was determined as before (Pietras *et al.*, 1994; Pegram *et al.*, 1997). In selected experiments, tumor growth delay was calculated by graphing the volume of each treatment group and calculating the number of additional days it took to reach 500 mm^3 compared with control (Teicher *et al.*, 1998). In all studies, toxicities of treatment regimens were estimated by following changes in animal body weight and the incidence of drug-associated deaths.

Tissue preparation and paraffin-embedded tissue sections

At the end of the experiments, mice were sacrificed, and tumors were excised, fixed in formalin and embedded in paraffin according to established procedures (Luna, 1968). Paraffin-embedded tumors were prepared in 4–6 μm sections, mounted on positively-charged Superfrost Plus slides (Fisher, Houston, TX, USA) prior to immunohistochemical staining, and sections were then deparaffinized in xylene, followed by 100, 95, 70 and 50% ethanol and rehydration in H_2O.

Quantification of tumor vessel counts

Histological sections of tumors from *in vivo* treatments of ovarian cancers were analysed for degree of angiogenesis by estimates of tumor-associated blood microvessel density. Tumor microvessel density was measured by counting the number of capillary blood vessels per high power field in sections stained with antibodies against von Willebrand Factor (vWF) as before (O'Reilly *et al.*, 1997). In brief, anti-human vWF/HRP antibodies (EPOS; Dako, Carpinteria, CA, USA) were applied on tissue sections to mark endothelial cells and were then detected using a specific substrate system. Vessels were counted by use of a light microscope.

Apoptosis assay

Histological sections of tumors from *in vivo* treatments of ovarian cancers were analysed for apoptosis using a detection system described previously (Ellis *et al.*, 1991; O'Reilly *et al.*, 1997). Apoptosis was assessed by a specific colorimetric detection system (Promega, Madison, WI, USA) (Ellis *et al.*, 1991; Steller, 1995). In brief, fragmented DNA of apoptotic cells were end-labeled using a modified TUNEL (TdT-mediated dUTP nick-end labeling) assay. Biotinylated nucleotide was incorporated at 3′-OH DNA ends using terminal deoxynucleotidyl transferase. Horseradish peroxidase-labeled streptavidin was then bound to biotinylated nucleotides and was detected using peroxidase substrate, hydrogen peroxide, and the stable chromogen diaminobenzidine. Using this procedure, apoptotic nuclei stained brown. An apoptotic index was estimated by the percentage of cells scored with a light microscope at 200-fold magnification (O'Reilly *et al.*, 1997).

Detection of VEGF in tumors

In selected *in vivo* experiments, human tumor xenografts were freshly excised and dissected free of mouse tissues. Human tumor tissue was then dissociated and homogenized *in vitro* by established methods (Pietras and Roberts, 1981). Total protein concentration in homogenates was assessed by BCA assay (Pierce Biochemical), and protein samples were separated on a 4–12% precast Tris-Glycine gel (Novex). Proteins were then transferred to a nitrocellulose membrane and subjected to immunodetection with anti-VEGF monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), using the ECL Western blotting system (Amersham Pharmacia) as before (Chazin *et al.*, 1992; Pietras *et al.*, 1994).

Statistical analysis

Statistical evaluation of data by *t*-tests and analysis of variance as appropriate was conducted using methods described before (Pietras *et al.*, 1994; Pegram *et al.*, 1997). All computations were made with Stat View and Super ANOVA software (Abacus Concepts, Berkeley, CA, USA).

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